(54) Title: ON-LINE PRODUCT IDENTIFICATION IN A CHROMATOGRAPHY EFFLUENT BY SUBTRACTION
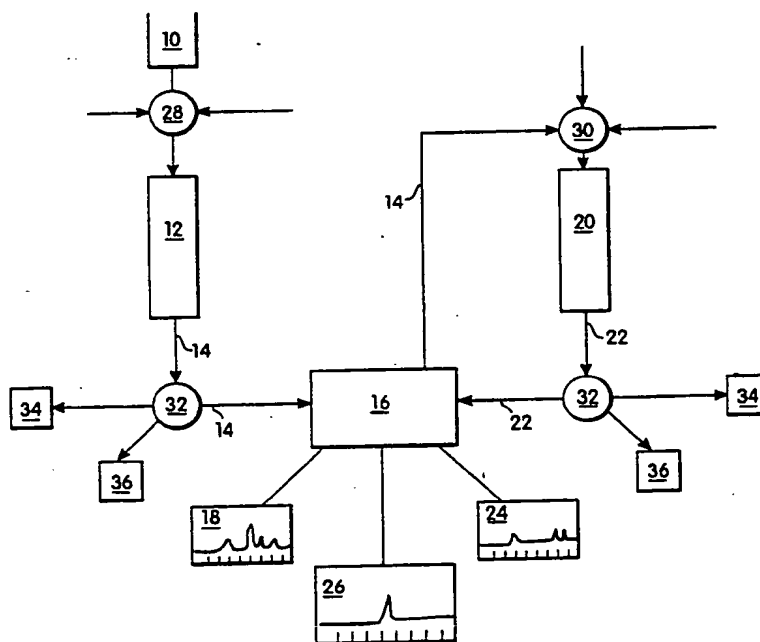
(57) Abstract

Disclosed is a method and apparatus for the rapid identification of a solute of interest in an effluent stream (14). The method involves separation of the solutes in the effluent (14) and identification f a particular solute of interest by its selective subtraction from the effluent stream (14). The effluent stream (14) from a first system (12) capable of partitioning the components of the mixture is passed through a detector (16) to produce a first output (18) which describes the temporal and/or spacial sequence of the mixture components exiting the first system (12). The effluent stream (14) is then passed through a second system (20) capable of selectively extracting a solute of interest from said effluent stream (14) and is then passed through a detector (16).

ON-LINE PRODUCT IDENTIFICATION IN A CHROMATOGRAPHY
EFFLUENT BY SUBTRACTION

The present invention is generally related to a
method and apparatus useful in chromatographic
procedures. More particularly, this invention relates
to a method for rapidly identifying products in a
chromatography effluent.

Background of the Invention

Chromatographic and electrophoretic techniques are
well known in the art as means for separating
components (solutes) present in a mixture. These
techniques are particularly useful in the chemical and
biotechnological arts. True chromatography describes
the separation of solutes according to their different
partitioning between two (or three) phases. The phases
generally are solid and liquid, and solute partitioning
results in their differing mobilities through a layer
of solid particles (matrix) in the presence of a
flowing phase. Solute transfer through the layer may
be along a pressure gradient, generally referred to as
"liquid chromatography". In contrast, electrophoretic
systems separate solutes on the basis of their
electrophoretic mobility, isoelectric point, and/or
differential migration through a size discriminating
matrix. Solute transfer in these systems is driven by
a voltage gradient from an applied electric field,
e.g., "electrophoresis".

Chromatographic matrices can separate components by any of a number of criteria, including size, electrical charge, hydrophobic interactions and/or specific affinity for the matrix or binding sites thereon. Because the components in the mixture will vary in their affinity for the matrix, their partitioning as they pass through the matrix separates the components so that they exit the matrix sequentially, separated temporally and spacially. Determination of the location of the various separated components, or of a given component of interest within the sequence, generally is achieved by collecting the fluid phase exiting the matrix (effluent stream) as a series of fractions and sampling these fractions to identify their contents by any of a number of means known in the art.

Resolution of the various components in the mixture depends on several considerations, chief among them being the partitioning ability of the matrix and the system's theoretical plate height and plate number (see infra). In general, a large surface area-to-volume ratio is desired. Matrices for liquid chromatography systems typically are housed in cylindrical chromatography systems known as columns. In electrophoresis systems, high resolution also demands efficient removal of the heat generated by the applied electric field. Capillary electrophoresis, or other electrophoretic modules which provide a large surface area-to-volume ratio, dissipate Joule heat well, allowing rapid analysis without significant loss of resolution.

The need to monitor a product's status during its synthesis or purification is well known in the art. Status monitoring is particularly important in multi-step preparative protocols. Frequently, the identity and, often, quality of a product in a mixture must be determined at each step. Product monitoring also may be used as part of a feedback system to adjust process parameters. Generally, identification is determined using a previously established criterion for identification, for example, a characteristic absorbance measured at a given wavelength. If the product of interest is a protein, identification also may be by molecular weight, activity, and/or immunoaffinity.

Unfortunately, product and/or process monitoring may be undesirably time-consuming, particularly when multiple samplings are required, as during a multi-step preparation. The time limitation of monitoring is of particular concern in industrial or other large scale preparations. To be useful, the monitor step should be rapid, adaptable, and repeatable. Accordingly, there exists a need for a method of rapidly identifying the presence and location of a molecule of interest during any preparative protocol.

It is therefore an object of this invention to provide a method and apparatus for the rapid, on-line identification of molecular products during a preparative protocol, such as during their synthesis or purification. Another object is to provide a method and apparatus for rapidly identifying the presence and

location of a solute in a chromatography effluent. Another object is to provide a method for rapidly detecting the concentration of a product in an effluent stream. Still another object is to provide a method for rapidly assessing the success of a purification or separation protocol. These and other objects and features of the invention will be apparent from the drawing, description, and claims which follow.

Summary of the Invention

A method and apparatus now has been discovered for
rapidly identifying the presence and location of a
preselected solute or subset of solutes in an effluent
stream. The method and apparatus are particularly
useful as part of a monitoring system during a
preparative protocol. As used herein, "differential
migration separation systems" (DMSS) is understood to
include all methods known in the art for separating
solutes present in a mixture, including those known as
"liquid chromatography" and "electrophoresis."

The method of this invention comprises the steps of
first passing a mixture through a system capable of
separating the components in the mixture (solutes) so
that they are separated temporally and spacially to
some degree as they exit the system in a fluid phase
(effluent stream). In preferred embodiments of the
invention, this first system is a liquid
chromatography matrix, e.g., a column, or other means
for separating solutes, such as an electrophoresis
module. The effluent stream from this first solute
separation system (referred to herein as "first
effluent stream"), then is passed through a detector to
produce a first output describing the sequence of the
solutes exiting the column. Identification of a
particular solute of interest within this sequence of
solutes is determined by passing this first effluent
stream through a second system capable of selectively
extracting the solute of interest from the fluid phase.
Except for its ability to extract the solute of

interest, this second system should be substantially inert, so that the sequence of solutes is essentially unaltered as the fluid phase passes through the second system, except that it will be substantially depleted in the component of interest. Preferably, the selective extraction occurs by some form of specific binding interactions between the matrix and the solute of interest. Particularly useful selective extraction systems include use of immunoadsorbents and immunoaffinity matrices.

The effluent stream exiting the second system then is passed through a detector to produce a second output which describes the sequence of the components exiting the second system. Because the second system selectively extracts the component of interest without significantly altering the temporal and/or spacial arrangement of the other solutes in the effluent stream, the difference between the first and second outputs can be used to determine the location in the effluent stream of the product of interest. Thus, the component of interest may be missing or depleted in the second output. Accordingly, a comparison of the two outputs will identify the location of the solute of interest in the first effluent stream.

The detectors used may be any means for molecule detection commonly used in the art. Currently preferred detectors include apparatus capable of monitoring the U.V. absorbance of a liquid, such as a spectrophotometer. In addition, both the first and second outputs may be produced by a single detector or,

alternatively, by separate detectors. Similarly, the
first and second outputs may be compared visually or
electronically. Electronic comparison may include
subtraction of the second output from the first output
to produce a third output presenting only the presence
and location of the component of interest in the first
effluent stream. The detector also may comprise means
for calculating and displaying the concentration of the
solute of interest in the first effluent stream. In
addition, the component of interest bound to the second
system subsequently may be eluted, detected and
quantitated as a means of confirming the data generated
by subtraction. Finally, the apparatus of this
invention may be integrated into an automated
purification or other preparative system, to act as a
product and/or process monitor, and the various steps
involved in performing the method of the invention
placed under computer control.

The method and apparatus of this invention are
rapid, reliable, and adaptable. The method of this
invention is particularly useful in the preparation of
biological macromolecules, particularly in the
separation and purification of proteins. It will be
understood by those skilled in the art that in a
particularly useful aspect of the invention, the first
and second systems may be readily regenerated with
recycling solvents, allowing the systems to be used
repeatedly throughout a given procedure.

The method and apparatus of this invention also may
be used to identify multiple components in an effluent
stream, by passing different samples of the first
effluent stream through different second systems
capable of selectively extracting different solutes of
interest and comparing the outputs from these systems
with the first output.

The method of this invention also may be used to
assess the purity of a solute of interest. Because the
second system is designed to selectively extract the
solute of interest from the first effluent stream, the
presence of any contaminants that coelute with the
solute of interest in the first effluent stream will be
indicated in the second effluent stream.

In still another embodiment of the invention, the
method and apparatus disclosed herein may be used as
part of an on-line process monitoring system to assess
process conditions in real time, with the information
generated used to alter conditions as needed to
optimize production. For example, coeluting solutes
identified by overlapping peaks in a monitoring output
on-line may be separated by altering particular process
conditions such as, for example, a buffer pH or the
parameters of an elution gradient.

Brief Description of the Drawings

Figure 1 is a schematic representation of one
embodiment of the apparatus of this invention;

Figures 2A-2C represent elution curves based on
absorbance at 280nm for a first output (2A), and a
second output (2B), wherein the solute of interest,
HSA, is missing in the second output, and 2C represents
the absorbance spectrum for the subsequently eluted
HSA;

Figures 3A-3C represent elution curves based on
absorbance at 280nm for a first and second output (3A
and 3B, respectively) and for HSA alone (3C); and

Figures 4A-4C represent elution curves based on
absorbance at 280nm for a first output (4A), and a
second output (4B), and 4C is an overlay of these first
and second outputs, showing the absence of the solute
of interest, IgG, from second output.

Detailed Description

The method and apparatus of this invention may be understood by referring to the schematic representation of one embodiment of the invention, depicted in Figure 1. A sample 10 containing the mixture to be separated is provided to a first system 12 capable of partitioning the components of the mixture (solutes). The effluent stream 14 from this first system containing the separated solutes then is passed through a detector 16 to produce a first output 18 which describes the temporal and/or spacial sequence of the mixture components exiting the first system. The effluent stream 14 then is passed through a second system 20 capable of selectively extracting a solute of interest from the first effluent stream 14. The temporal and spacial sequence of the solutes in the second effluent stream will be substantially identical to that of the first effluent stream, provided that the second system is of an appropriate geometry and is sufficiently inert such that all but the component of interest pass through the system without significant interaction or delay.

The effluent stream 22 exiting the second system 20 then is passed through a detector 16 to produce a second output 24 describing the temporal and/or spacial sequence of the solutes remaining in the effluent. The detector also may subtract the second output 24 from the first output 18 to produce a third output 26 describing only the presence and position of the solute of interest in the first effluent stream 14. The

detector also may have means for determining the
concentration of the solute of interest in the first
effluent stream, and means for displaying this data.
Finally, the bound solute may be subsequently eluted
from the second system, detected, and quantitated to
confirm the substraction data.   Where the solute of
interest is a protein, a typical detector is a
conventional detector which measures U.V. absorbance
through a film of fluid.

The apparatus may be used as part of a monitoring
system within a molecule preparation system, which may
be automated. In this case the apparatus preferably
further comprises a multi-port sampling valve 28 such
as is found in automated protein production systems
known to those skilled in the art. The multi-port
sampling valve may provide samples to the first system
12, as well as all necessary solvents or buffers,
including washing solvents, eluting solvents, "running"
solvents for electrophoresis systems, and recycling
solvents to regenerate the system as needed between
samplings. Similarly, a multi-port sampling valve 30
provides fluids to the second system, including the
first effluent stream and all necessary solvents.
Valve position for both multi-port valves preferably is
under computer control, and fluid delivery may driven
by a metering pump. The multi-port valves further may
include "stream splitters" or other means for reducing
and/or directing only the desired flow rate to the
first and second systems. Valves 32 at the exit of
both the first and second systems direct the fluids
exiting the systems to a detector 18, to waste

collectors 34, or to product collectors 36. If
desired, means also may be provided for recycling the
detected samples. It will be understood by those
skilled in the art that, where the first and/or second
system is an electrophoretic module, means for
providing the necessary applied electric fields also
will be provided and may be under computer control.

As part of a process monitoring system, the method
of this invention may be useful in assessing and/or
developing a particular separation or purification
protocol. For example, in an ion exchange
chromatography system, variations in pH significantly
affect solute separation. Using the method of the
present invention one can rapidly assess the effect of
various pH values on solute separation on-line, and
alter appropriate conditions to optimize separation.
In preferred embodiments of the invention, this
assessment can be performed rapidly using minute sample
quantities. Accordingly, the method allows on-line
production optimization without substantial loss of
sample or time.

As part of a product monitoring system, the method
of this invention also may be useful in identifying the
presence of contaminants that coelute with the solute
of interest. Provided that the second system
selectively extracts essentially all of the solute of
interest from the fluid phase without significantly
affecting the quantity or position of the remaining
solutes in the sample mixture, the presence of a solute
in the second output at the position generally occupied

by the solute of interest can indicate the presence of
a contaminant. Moreover, identification can be
corroborated by eluting the solute of interest,
quantifying it and comparing this value with that for
the pertinent peaks in the first and second outputs.

Among the key features of this invention which make
it useful as part of a product and/or process
monitoring protocol are the speed, quality, and
reliability of solute identification. This requires
rapid fluid transfer through both systems in the
apparatus, and no significant loss of resolution
between the first and second effluent streams.

Resolution of partitioned solutes in a mixture is a
function of both the affinity of the various solutes
for the partitioning component (generally a matrix) and
the theoretical plate height of the system. A "plate"
in column chromatography can be considered to be the
largest uniform zone able to accommodate a solute. The
smaller the plate height of a column, the more discrete
steps (higher plate number) a solute will encounter
traveling through the matrix, providing better
separation between similar components. Generally, the
greater the matrix surface area-to-column volume ratio,
the smaller the plate height and larger the plate
number achievable. Column design generally focuses on
designing the smallest matrix volume possible that
provides a sufficient plate number to resolve
components of interest. Smaller volumes increase the
speed of fluid transfer through the system and reduce
zone spreading. Preferred matrices are those composed

of porous particles, as these provide a substantially greater surface area-to-volume ratio than a packed matrix of solid (non-porous) particles.

One particularly useful differential migration separation system in use today is the HPLC system (high performance liquid chromatography). HPLC columns utilize matrices of homogenous porous small bead particles. Because the dense packing of these small beads creates a high resistance to liquid flow, the equipment is designed to operate at high pressures, which allows rapid fluid transfer. The densely packed particles create a large surface area-to-volume ratio which works well resolving small molecular weight solutes. However, conventional HPLC systems are substantially less successful when used to resolve large molecular weight solutes such as proteins. Flowthrough speed of large molecular weight solutes such as proteins through a conventional HPLC matrix becomes limiting, primarily because mass transfer within the particle pores becomes diffusive, as compared to the mass transfer between pores, which is convective. While one can increase flow rates at the expense of high pressure drops, this tends to reduce separation quality.

These limitations of conventional HPLC analysis are overcome by the use of high speed chromatographic matrices capable of perfusive chromatography. These matrices comprise particles which may be of the same overall size as are sometimes employed in conventional matrices, but having increased intraparticle porosity. In addition to intraparticle throughpores of increased

diameter, e.g., 6000-8000 Å, particles capable of
perfusive chromatography have a network of 500-1500 Å
pores interconnecting the larger throughpores. The
resulting network limits the diffusional path lengths
within the particles so that mass transfer within the
particle pores over a large fluid velocity range is
governed essentially by convection. The effect is to
permit increase of the mobile phase velocity of these
systems to greater than 10-100 times that of
conventional HPLC systems (greater than 1000 cm/hr),
with no substantial loss in resolution. A more
detailed description of perfusive chromatography is
provided in U.S. Application No. 376,885, filed July 6,
1989 and Afeyan et al. (1990) Bio/Technology 8:203-206,
the disclosures of which are hereby incorporated by
reference. Perfusive chromatography matrix materials
are available commercially from PerSeptive Biosystems,
Inc., of Cambridge, MA U.S.A. The increased porosity
of particles capable of perfusive chromatography
substantially increases the available surface area of
the column, typically to levels within the range of
about 30-50 m^2/ml, greatly reducing column plate
height. Accordingly, miniscule columns (microcolumns)
may be used and analysis may be performed at heretofore
unattainable speeds with no significant loss of
resolution.

Perfusive matrices are currently preferred liquid
chromatography matrices for both the first and second
system in the apparatus of this invention. Perfusive
matrices for use in the first system, designed to
partition and resolve components of a mixture, may be

derivatized as desired using conventional methods known
to those of ordinary skill in the art, to create a
particular chromatography system. For example, the
matrix may partition solutes by size, or be derivatized
to separate solutes by charge (e.g., act as ion or
anion exchangers), by metal ion affinity, or by
hydrophobicity or hydrophilicity. Other useful
matrices include inorganic substances such as calcium
phosphate (hydroxyapatite), bentonite, alumina, and
titanium or zinc oxide gels.

Another method for separating solutes in solution
and particularly useful in the method of this invention
is electrophoresis. Capillary electrophoresis, in
particular, provides the appropriate geometry (high
surface area-to-volume ratio) needed to dissipate the
Joule heat generated by high applied electric fields.
By tolerating these high applied fields, the capillary
electrophoresis system, like perfusive HPLC, allows
rapid throughput without loss of resolution. In
addition, the capillary geometry can achieve the
necessary plate number in a small volume, allowing the
system to be run as a microcolumn. Accordingly, as
with perfusive HPLC systems, capillary electrophoretic
systems allow significant sample size reductions and
rapid analysis.

Electrophoresis can separate molecules by a number
of different modes, and these all may be performed in a
capillary system. Among the most useful modes are zone
or "free-flow" ("open") electrophoresis, gel
electrophoresis and isoelectric focusing. Zone
electrophoresis is characterized by an absence of solid

supports and separation is within a single phase (e.g.,
liquid). Nonetheless, the separation record in zone
electrophoresis, particularly capillary zone
electrophoresis, still resembles the record obtained
for standard elution chromatography and the formal
concepts of plate number and resolution as defined for
column chromatography are widely accepted.

In general, any electrophoretic system, including
conventional polyacrylamide "slab" gels, may be used,
provided the limitations that zone broadening imposes
on the system (e.g., caused by diffusion, Joule heat
and/or changes in conductivity) are understood and
minimized. For example, isoelectric focusing and zone
electrophoresis systems having channel thicknesses less
than about $200 \mu m$ generally are considered useful for
the method and apparatus of this invention. Further
information on electrophoretic theory, applications,
instrumentation and automation, including capillary
electrophoresis, can be found in a number of sources
known to those of ordinary skill in the art.
Particularly useful sources include Karger et al.,
(1989) J. Chromatogr. 492:585-614; Foret et al., (1990)
Electrophoresis 11:661-664; and Novotny et al. (1990)
Electrophoresis 11:735-749.

As stated above, the second system should not
significantly affect the position or concentration of
the solutes remaining in solution. This means that the
geometry of the system is important. The minimum
volume that will adequately bind substantially all of
the solute of interest should be used. In addition,

non-specific binding must be minimized to prevent false
negatives. Preferably, non-specific adsorption should
be less than about 1 ng/10 ul. Accordingly, the
binding surface or matrix should be substantially
inert, capable of selectively extracting the solute or
solutes of interest, preferably quantitatively, without
significantly altering the resolution of the solutes
remaining in the effluent. If desired, non-specific
binding may be further minimized in the second system
by first coating the potential non-specific binding
sites with a nonspecific molecule, before loading the
sample. It will be understood by those skilled in the
art that this "coat" molecule should bind sufficiently
under elution conditions so as not to interfere with
the output of the second effluent stream.

Currently preferred matrices for selectively
extracting the solute of interest are those capable of
specific binding interactions with the solute. Most
preferably, these interactions are reversible, and the
system may be regenerated by means of one or more
recycling solvents capable of dissociating the solute
from the column, and preparing the system for another
sample. Useful solute-specific binding sites include
immunoadsorbents (e.g., antibodies) and other proteins
capable of interacting specifically with the solute of
interest. For example, one can envision the solute and
solute-specific binding site comprising any
ligand/enzyme combination, including hormones, toxins,
lectins and their appropriate receptors. Where the
solute of interest is an enzyme, the binding site may
comprise a pseudo-substrate or an inhibitor. In

general, the solute-specific binding site (solute-
specific affinity sorbent) can be any immobilized
ligand that demonstrates a bioaffinity for the given
eluent of interest. The matrix surface may be
derivatized so that the solute-specific binding site is
bound irreversibly to the matrix surface. The bound
solute then maybe eluted and the column regenerated for
subsequent samples. Alternatively, the solute-specific
binding site may be attached non-covalently to the
matrix surface. This allows the system to be adapted
for use with different target solutes. One particular
solute-specific binding site may be removed from the
system by means of one or more recycling solvents, and
a second binding site, specific for a second, different
solute, then applied to the system. For example,
protein A or protein G may be covalently bound to the
matrix surface, allowing multiple, different
solute-specific antibodies to be bound to the matrix in
turn.

The solute-specific matrix may be incorporated in a
liquid chromatography system or in an electrophoretic
system (e.g., capillary electrophoresis system).
Alternatively, the solute-specific binding sites may be
attached to the inner surface of a capillary tube, or
any other surface capable of providing a sufficiently
high surface area-to-volume ratio. Capillary
chromatography then may be performed using a pressure
gradient or a voltage gradient.

The invention may be further understood by the
following, nonlimiting examples:

Example 1.

. In this experiment, human serum albumin (HSA) is
identified in a mixture. A first system is created
using a perfusive ion exchange media (IEC, POROS SM,
4.6 x 50 mm column) to separate a mixture containing
human serum albumin (HSA, 50μg); chymotrypsin (CT,
25μg); cytochrome C (Cyt, 25μg); and lysozyme (Lys,
25μg) in 1 ml of phosphate buffered saline (PBS). The
mixture is loaded at 1 ml/min in PBS, and eluted at 1
ml/min with a gradient of 90% Buffer A (20mM morpholine
ethane sulfonic acid, pH 6.0)/10% Buffer B (20mM MES,
containing 1M NaCl) at t=0 minutes, and 50%
Buffer A/50% Buffer B at t=8 minutes. The first
effluent stream is detected with a spectrophotometer
measuring absorbance at 280 nm. As can be seen in
Fig 2A, five peaks are identified in the first output.

The first effluent stream then is passed through
the second system which is placed in line after the IEC
column. The second system is created with a perfusive
reverse phase media (POROS RM, 2.1 x 30mm) that has
previously been loaded at 1 ml/min with a solution
containing 170 μg purified anti-HSA (Sigma Co,
Cincinnati, OH, No. A-2672) and 1.6 mg bovine serum
albumin (BSA). BSA is added to essentially coat the
unbound matrix surface, thereby substantially
preventing non-specific binding of the sample with the
matrix. As seen in Fig 2B, when the first effluent
stream is passed through the second system, at least
90% of peaks 1 and 2 is depleted, whereas peaks 3, 4
and 5 elute with their concentrations unaffected.

Moreover, peaks 3, 4, and 5 elute from the second
system at the same relative distance from one another
as they did from the first system. As a check, the
bound HSA then is eluted from the second system at
1 ml/min with a solution containing 2% acetic acid and
300 mM MgCl$_2$. As can be seen in Figure 2C, this
releases at least 90% of the bound HSA in a broad peak
at approximately 13 min. For this and the subsequent
experiments the characteristic outputs for each member
of the mixture alone has previously been determined for
the conditions of the given experiment. In this first
example, then, it is known that, in the presence of PBS
and at a flow rate of 1 ml/min., HSA elutes at about 1
min., CT at about 2.3 min., Cyt at about 2.9 min., and
Lys at about 4.7 min. However, it should be clear from
the Specification and Examples that this verification
is not necessary in the method of this invention.

Example 2.

        A second method for identifying HSA is tested here.
A sample containing 45 μg HSA, 22.5 μg CT, 22.5 μg Cyt
and 22.5 Lys is passed through a first system
containing a perfusive ion exchange media (POROS SM,
4.6 x 50 mm column) under the same conditions as for
Example 1. The first output is shown in Fig. 2A. In
this example, somewhat better separation is achieved
and HSA appears to elute as a single peak (at 1.1 min).
In addition, a small amount of protein appears in the
void fraction at 0.58 min.

The first effluent then is passed through a second
system in line after the POROS SM column. The second
system is created with a perfusive protein G affinity
column (POROS GM, 2.1 x 30 mm) which previously has
been loaded at 0.5 ml/min with 2 ml PBS containing
polyclonal goat anti-HSA (1 ml, Sigma Co., No. A-7544,
3.9 mg total antibody/1 ml). Elution is performed as
for Example 1 and the second effluent then is detected
at 280 nm, producing the output represented in Fig. 2B.
A comparison of Figs. 2A and 2B show that at least
about 90% of the HSA present in the first effluent is
captured by the second system without affecting the
remaining solutes, unequivocally identifying the
presence of HSA in the sequence.

To determine if the protein in the void fraction in
Example 2 is HSA, HSA is run over an IEC column alone,
under the same conditions as described for Examples 1
and 2 (100 μg HSA in PBS, loaded in 20 μl). Elution
with the standard Buffer A/Buffer B gradient described
above produces both the void fraction peak at 0.58 min
and HSA (at 1.1 min, see Fig. 2C). This chromatogram
clearly shows that the protein in the void volume is a
contaminant introduced with HSA.

Example 3.

In this example, IgG is the solute of interest and
comprises a small portion of a crude cell extract
supernatant mixture.

A first system, containing a perfusive anion exchange matrix (POROS QM, 4.6 x 100 mm column) is loaded with a 50 μl sample volume containing 50 μg IgG and a 100 fold excess of a hybridoma crude cell culture supernatant (5 mg protein). The sample is loaded at 5 ml/min and eluted at the same flow rate with a gradient of 100% Buffer C (20 mM Tris, O NaCl, pH 8)/0% Buffer D (Buffer C + 1M NaCl) at t=0 minutes; and 0% Buffer C/100% Buffer D at t=10 minutes. The effluent is detected at 280 nm to produce the output represented in Fig. 4A.

The effluent then is passed through a second system in line with the first system. The second system contains a perfusive Protein A affinity matrix (POROS AM, 4.6 x 50 mm column). The sample is run at 5 ml/min and this second effluent is detected at 280 nm to produce the output represented in Figs. 4B. The third peak, IgG, at 2.1 min. is missing from the second output. An overlay of Figs. 4A and 4B, represented in Fig. 4C, shows that the position of the remaining solutes is unchanged in the first and second effluents. IgG can be eluted from the column with a recycling solvent composed of 2% acetic acid, 0.3M $MgCl_2$ and 0.15 M NaCl.

The invention may be embodied in other specific forms without departing from the spirit and central characteristics thereof. The present embodiments are therefore considered in all respects to be illustrative and not restrictive, the scope of the invention being indicated by the pended claims rather than by the

foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

What is claimed is:

Claims:

1.   An apparatus for separating solutes
comprising:

        means for interacting differentially with
plural solutes in a liquid feed to produce a first
stream rich sequentially in individual solutes;

        first detector means for providing a first
output indicative of the temporal presence of said
individual solutes in said stream;

        means for extracting from said stream a
preselected subset of said individual solutes and to
produce a second stream rich sequentially in remaining
individual solutes;

        second detector means for providing a second
output indicative of the temporal presence in said
second stream of said remaining individual solutes; and

        comparator means for comparing said first
output and said second output thereby to enable
identification of the location of said preselected
subset of individual solutes in said first stream.

2.   The apparatus of claim 1 wherein said first
detector means and said second detector means comprise
a single detector.

3.   The apparatus of claim 1 wherein one of said
detector means comprises means for monitoring the
ultraviolet absorbance of solutes in a liquid.

4.   The apparatus of claim 1 wherein said means
for interacting differentially with plural solutes in a
liquid feed comprises a liquid chromatography system.

5.   The apparatus of claim 4 wherein said liquid
chromatography system comprises chromatography media
selected from the group consisting of anionic exchange,
cationic exchange, hydrophobic interaction, hydrophilic
interaction, molecular sieve, size exclusion, metal ion
affinity and bioaffinity media.

6.   The apparatus of claim 1 wherein said means
for interacting differentially with plural solutes in a
liquid feed comprises an electrophoretic system.

7.   The apparatus of claim 6 wherein said
electrophoretic system is a capillary electrophoretic
system.

8.   The apparatus of claim 6 or 7 wherein said
electrophoretic system is selected from the group
consisting of gel electrophoresis, zone electrophoresis
and isoelectric focusing.

9.   The apparatus of claim 1 wherein said means
for extracting comprises a solute-specific affinity
sorbent.

10.   The apparatus of claim 1 wherein said plural
solutes in said first liquid feed comprise biological
macromolecules.

11.   The apparatus of claim 1 wherein one of said
detector means comprises means for detecting the
presence of a protein in said stream.

12.   The apparatus of claim 1 wherein one of said
detector means comprises means for detecting the
instantaneous concentration of said individual solutes.

13.   A method of identifying the location of a
preselected subset of solutes in an effluent stream
from a solute separation apparatus, said method
comprising the steps of:

passing said effluent stream through a
detector to produce a first output indicative of the
temporal presence of individual solutes in said stream;

thereafter, passing said effluent stream
through means for extracting a preselected subset of
solutes from said effluent stream;

thereafter, passing said effluent stream
through a detector to produce a second output
indicative of the temporal presence of remaining
individual solutes in said effluent stream; and

comparing said first output with said second
output to determine the location of said preselected
subset of solutes in said effluent stream.

14.   The method of claim 13 wherein said means for
extracting comprises an apparatus containing a solute-
specific affinity sorbent.

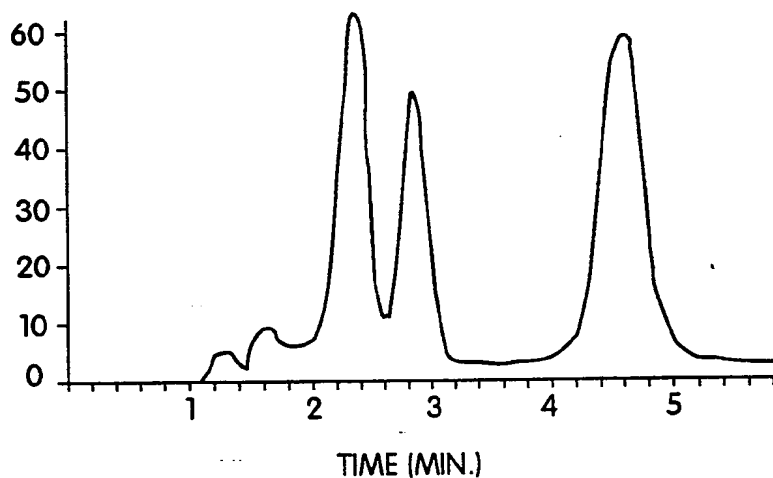15. The method of claim 13 wherein the comparing step is conducted electronically.

Fig. 1

Fig. 2A

Fig. 2B

Fig. 2C

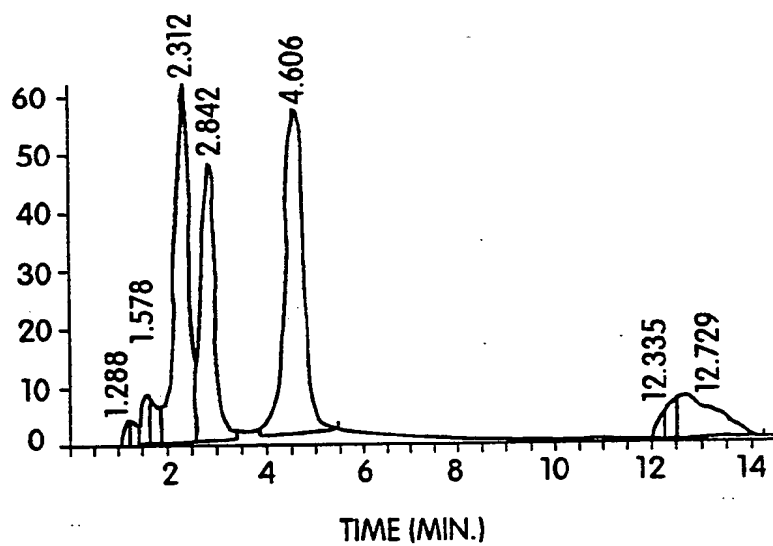Fig. 3A

Fig. 3B

Fig. 3C

Fig. 4A

Fig. 4B

Fig. 4C

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): B01D 15/08
U.S. Class: 210/656, 93,198.2

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
	210/635,656,85,93,198.2,73/61.52,61.55,61.58;422/70,436/161, 162

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US,A, 3,686,117 (Lauer) 22 August 1972 see column 3, lines 25-28 and column 4, lines 7-11.	1-12
Y	JP,B, 61-212 759 (Shimadzu Corp) 20 September 1986, see English Abstract, lines 1-5.	13-15
Y	Synder and Kirkland, Introduction to Modern Liquid Chromatography, John Wiley and Sons, Inc., New York 1979, pages 728-729, see page 728, lines 40-41 and page 729, lines 1-2.	

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

26 June 1992

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

28 AUG 1992

Signature of Authorized Officer

Ernest G. Therkorn

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